Trends in Immunology

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Review

The Contribution of Epigenetics to Cancer Immunotherapy

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Effective anticancer immunotherapy treatments constitute a qualitative leap in cancer management. Nonetheless, not all patients benefit from such therapies because they fail to achieve complete responses, suffer frequent relapses, or develop potentially life-threatening toxicities. Epigenomic signatures in immune and cancer cells appear to be accurate and promising predictors of patient outcomes with immunotherapy. In addition, combined treatments with epigenetic drugs can exploit the dynamic nature of epigenetic changes to potentially modulate responses to immunotherapy. Candidate epigenetic biomarkers may provide a rationale for patient stratification and precision medicine, thus maximizing the chances of treatment success while minimizing unwanted effects. We present a comprehensive up-to-date view of potential epigenetic biomarkers in immunotherapy and discuss their advantages over other indicators.

Predicting Immunotherapy Success or Failure: The Importance of Developing Reliable 'Biomarkers'

Immunotherapy (see Glossary) has become a game changer in cancer treatment. It relies on enhancing the patient's immune defenses to combat tumor cells. However, the significant proportion of nonresponding cases and treatment-associated toxicities remain an obstacle to therapeutic success. Therefore, it is important to discover and develop good predictors of therapy efficacy and/or toxicity such that treatments can be personalized and the chances of a cure increased. We discuss in depth the latest research on epigenetic marks as biomarkers for immunotherapy, their potential and limitations, and the preclinical and clinical studies currently being conducted; we provide comprehensive and novel insights into their present and future usage in the clinic.

Cutting-Edge Immunotherapy-Based Strategies and Their Limitations

Immunomodulatory agents targeting the interferon (IFN)-α2b pathway or the interleukin (IL)-2/IL-2R axis (aldesleukin) were approved by the US FDA in 1986 and 1992 for the treatment of leukemia and melanoma, respectively. Subsequently, the monoclonal antibody (Ab) rituximab, which targets B cell CD20, was approved by the FDA for non-Hodgkin lymphoma. Since then, Ab-based therapies have substantially improved with the development of **bispecific and trispecific antibodies** [1] that may allow more precise and effective treatment strategies. Therapeutic vaccination against cancer also emerged as a promising immunotherapeutic strategy at the beginning of the 21st century, leading to FDA approval of the dendritic cell (DC)-based vaccine sipuleucel_T for the treatment of prostate cancer in 2010. In this context, the arrival of **immune checkpoint blockade (ICB)** therapy was a tipping point that created new possibilities for clinical applications.

Since FDA approval in 2011 of the cytotoxic T-lymphocyte antigen 4 (CTLA-4) targeting ICB ipilimumab to treat metastatic melanoma, many immunotherapeutic approaches targeting immune checkpoint molecules have been included in clinical practice guidelines for cancer treatments. Pembrolizumab and nivolumab [anti-PD-1 (programmed cell death) monoclonal antibodies (Abs)]

Highlights

Recent immunotherapy treatments are showing promising results in cancer management, but a proportion of patients do not fully benefit from therapy and fail to achieve durable responses or experience relapse.

Chimeric antigen receptor (CAR) vector insertion at epigenetic chromosomal sites may determine the potency and durability of CAR-T cell cytotoxic responses, as shown for the complete remission of a chronic lymphocytic leukemia (CLL) patient with *TET2* disruption caused by CAR19 vector integration.

Novel biomarkers of response are needed, and epigenetic biomarkers may represent solid candidates for filling this niche, thereby contributing to theragnosis and precision medicine.

Combinatorial approaches of epigenetic drugs with immunotherapy may synergize to reshape the tumor microenvironment and restore an effective antitumor response, thus overcoming immunotherapy limitations for some cancer types.

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were approved by the FDA in 2014 and 2015, respectively, for the treatment of various solid and hematological malignancies. PD-L1 blockers atezolizumab and avelumab were FDA-approved in 2017 and durvalumab in 2018 for the treatment of bladder, lung, and Merkell cell carcinoma. One of the latest FDA-approved ICB molecules is the anti-PD-1 monoclonal Ab cemiplimab for metastatic cutaneous squamous cell carcinoma. Other promising immune receptors such as LAG-3, TIM-3, TIGIT, VISTA, OX40, CD40, 4-1BB, and GITR are currently under investigation in preclinical studies [2].

The success of immunotherapy success has extended to adoptive cell therapies with the approval of **chimeric antigen receptor (CAR) T cell therapy** by the FDA, followed by other promising strategies such as **tumor-infiltrating lymphocytes (TILs)** and **T cell receptor (TCR)-engineered cells** that are currently being tested in various advanced clinical trials for different malignancies (clinicaltrials.gov). Novel CAR-based strategies using natural killer cells (CAR-NK) in conjunction with lymphodepleting chemotherapy are also showing encouraging results in a single-arm dose-escalation Phase I/IIa trial (NCT03056339[i], recruiting) in which 73% of patients with non-Hodgkin's lymphoma or chronic lymphocytic leukemia (CLL) responded to treatment [3].

Nonetheless, beyond **tumor intrinsic resistance** to ICB, there are innate and adaptive mechanisms that affect antitumor-specific T cell activation, expansion, and **exhaustion** [2,4], leading to response failure. In addition, treatment can be associated with potentially life-threatening autoimmune-like toxicities leading to specific organ damage [5]. In general, the incidence of immune-related adverse effects (irAEs) is higher with combined therapies than with anti-PD-1/anti-PD-L1 Ab (66%) or anti-CTLA-4 Ab monotherapies (72%) [5]. In addition, combined blockade showed a higher fatality rate (1.23%) than anti-PD-1/anti-PD-L1 Abs (0.37%) and CTLA-4 blockers (1.08%) administered separately, according to a meta-analysis conducted in 19 217 ICB-treated patients with 25 types of solid cancers, mainly melanoma and lung cancer [6]. Severe-grade toxicities are mostly associated with anti-CTLA-4 therapies, where colitis is the primary cause of irAES-related death, affecting 8–22% of patients with melanoma treated with anti-CTLA-4 Ab [7]. Pulmonary toxicity (5% with anti-PD-1/anti-PD-L1 Abs, or combined treatment with anti-CTLA-4 Ab) and hepatitis incidence (3–9% with anti-CTLA-4 Abs) are low, but with potentially fatal consequences, mainly in melanoma and lung cancer [5]. A meta-analysis of 38 clinical trials carried out in different solid cancers showed that endocrine affectations such as pituitary or thyroid toxicity can appear after CTLA-4 or combined therapy, leading to gonad dysfunction [5]. In the case of CAR-T cell therapy, severe adverse toxicities, including **cytokine-release syndrome** [8], and **CAR-T cell-related encephalopathy syndrome (CRES)** [9], are more frequent. Thus, beyond most clinical indications and guidelines, it is essential to develop new biomarkers that will enable us not only to predict the intensity and duration of a clinical response (if any) but also the risk of developing important adverse effects, and thereby rationalize the use of these expensive customized therapeutic strategies.

Epigenetic Biomarkers: Pros and Cons

Molecules that act as predictors of the cancer response to immunotherapy (termed biomarkers) are commonly used in personalized cancer immunotherapy: these include PD-L1 expression [10], tumor-associated antigens (TAAs) [11], HLA expression [12], **TCR repertoire** assessments [13], **tumor mutational burden** and neoantigen identification [14], **mismatch repair deficiency** [15], the presence of TILs [16], and the presence of cells within the **tumor microenvironment (TME)** that might potentially inhibit antitumor immune responses [e.g., **regulatory T cells (Tregs)**, **tumor-associated macrophages (TAMs)**, and/or **polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs)**] [4,17], as well as the incidence of genetic alterations [18] that are associated with treatment response. The epigenetic control of these events has been

Glossary

Apoptosis: a highly regulated programmed cell death mechanism that occurs in the absence of cell membrane breakage, ensuring the correct disposal of dysfunctional cells.
Bispecific and trispecific antibodies: genetically engineered monoclonal immunoglobulins that respectively target two or three antigens/epitopes simultaneously.
CAR-T cell-related encephalopathy syndrome (CRES): neurotoxic side-effect developed in some patients following CAR-T cell infusion.
Chimeric antigen receptor (CAR) T cell therapy: an immunotherapy approach in which T cells are genetically engineered in the laboratory to recognize targeted antigens expressed in tumor cells.
CpG islands: genomic regions enriched in cytosine and guanine dinucleotides; CpG islands are located in DNA regulatory regions such as promoters.
Cytokine-release syndrome (CRS): a systemic inflammatory response that is triggered, for example, by CAR-T cell activation following targeted antigen recognition.
Deconvolution analysis: a computational method to infer the proportion of components and their relative contributions to a particular signal from a complex sample.
Epigenetic biomarker: a modification or signature used as a qualitative and/or quantitative putative indicator of physiological/pathological conditions, or in response to treatment.
Epigenetic drug: targets epigenome-related pathological changes to allow the treatment of several disorders, including cancer.
Epigenetic fingerprints: molecular signatures comprising sets of epigenetic modifications relative to a particular condition.
Exhaustion: a state of cell dysfunction accompanied by progressive loss of effector function that occurs after persistent antigen stimulation.
Immune checkpoint blockade (ICB): an immunotherapy that abolishes inhibitory signals in tumor-associated immune cells, thus supporting an effective anticancer T cell response.
Immunological synapse: contact between an antigen-presenting or target cell and a lymphoid lineage cell that orchestrates lymphocyte activation and a subsequent immune response.

extensively demonstrated, supporting the relevance of using specific epigenetic changes (Box 1) as potential biomarkers for immunotherapy. Cancer cell-intrinsic epigenetic alterations have been associated with carcinogenesis [19,20], tumor progression [21,22], and treatment resistance as a result of **lineage switch** in B cell lymphoma and leukemia [23]. Finally, treatment itself may cause epigenetic alterations, as evidenced from DNA methylation of relevant genes including *CD96*, *HHLA2*, *CCR5*, *CXCR5*, and *CCL5* in $CD8^+$ T cells purified from healthy donors following immunotherapy [24] (see Outstanding Questions).

Epigenetic biomarkers may offer additional advantages, such as low patient invasiveness, given that changes in DNA methylation can be measured in liquid biopsies and body fluids [25]. They can also convey a layer of information about the life habits and conditions of patients, and reveal details about the origin and evolution of a given disease [26]. In some instances, as discussed later for lung cancer [27], they can serve as diagnostic, prognostic, predictive, and therapy-monitoring indicators, facilitating the fostering of theragnosis and precision medicine [26]. Despite the great potential of epigenetic modifications as putative biomarkers, some caveats

Box 1. Epigenetic Modifications: DNA Methylation, Histone Modifications, and Noncoding RNAs

The term epigenetics comprises mitotically heritable changes that affect gene expression without modifying the primary genome DNA sequence. Epigenetic modifications include DNA methylation, modification of histone tails, and noncoding (nc) RNAs. Epigenetic alterations are key in directing the aberrant expression of tumor-associated genes that drive cellular malignant transformation and cancer progression.

DNA Methylation

The most abundant mark on DNA occurs via addition of a methyl (CH_3) group by DNA methyltransferases (DNMTs) at the 5′ position on the pyrimidine ring of cytosine residues in the context of a CpG dinucleotide. DNA methylation affects transcription factor binding and controls accessibility to regulatory regions in the DNA, modulating gene expression [85]. Aberrant modifications of 5-methylcytosine (5mC) profiles can promote uncontrolled cell proliferation and survival, as well as supporting tumor growth. Silencing of tumor-suppressor genes through promoter hypermethylation is a hallmark of many cancer types [86]. Removal of 5mCs occurs by sequential oxidation by TET enzymes (in dividing mammalian cells), generating 5-hydroxymethylcytosine (5hmC), 5-carboxycytosine (5caC), 5-formylcytosine (5fmC), and unmethylated C. Accumulating evidence suggests that oxidized intermediate forms could represent *bona fide* epigenetic marks [87,88].

Post-translational Histone Modifications

These control gene expression by modulating chromatin conformation and accessibility. Families of histone-modifying enzymes target specific residues at histone tails in response to external signals. The most frequent histone modifications include acetylation, methylation, and phosphorylation, but others including citrullination, ubiquitination, ADP-ribosylation, deamination, formylation, *O*-GlcNAcylation, propionylation, butyrylation, crotonylation, proline isomerization, and the recently discovered lactylation have also been described [89].

Acetylation. This mark consists of the reversible addition of an acetyl group (-CH_3CO) to lysine residues and is associated with gene transcription activation. Histone acetyltransferases (HATs) are chromatin writers that add acetyl groups, whereas histone deacetylases (HDACs) are erasers that remove this mark.

Methylation. The addition of methyl groups (-CH_3) to lysine and/or arginine residues in histone tails is catalyzed by several histone methyl transferases (HMTs), whereas histone demethylases (HDMs) catalyze removal. Depending on the specific modified residue, methylation marks can be categorized as activating (e.g., H3K4me3) or repressive (H3K9me3, H3K27me3).

Phosphorylation. Histone phosphorylation occurs mainly at serine (S), threonine (T), and tyrosine (Y) residues of histone tails and is positively associated with transcription and an accessible chromatin conformation. Histone H3 phosphorylation at tyrosine 41 (H3Y41) is enriched at active promoters close to transcription start-sites (TSS) together with the H3K4me3 mark.

ncRNAs

ncRNAs represent ~90% of human genome-derived RNAs, and include several different types of ncRNA that are classified according to size. ncRNAs >200 bp in length include long ncRNAs (lncRNAs), pseudogene transcripts, and circular RNAs (circRNAs). miRNAs, tRNA-derived small RNAs (tsRNAs), and PIWI-interacting RNAs (piRNAs) are ncRNAs <200 bp in length. Dysregulation of ncRNA expression contributes to the pathogenesis of many diseases including cancer [90].

Immunotherapy: a therapeutic intervention aiming to enhance the patient's own immune system.
Lineage switch: a conversion phenomenon affecting hematopoietic lineages: one cell lineage transforms into a different lineage that exhibits distinct lineage-defining markers.
Mismatch repair deficiency: a cellular defect in mismatch repair (i.e., genetic mutations in the machinery that corrects mistakes due to DNA replication), resulting in the accumulation of mutations by the cell.
Polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs): immune cells involved in an immunosuppressive response.
Proto-oncogenes: genes that regulate cell proliferation and differentiation; can potentially drive cancer when mutated or aberrantly expressed.
Regulatory T cells (Tregs): a subpopulation of $CD4^+$ T cells that dampen the immune response, preventing autoimmune diseases or promoting tumor progression by suppressing antitumor responses.
T cell receptor (TCR)-engineered cells: cells encoding an engineered TCR that has been inserted as part of an immunotherapeutic strategy.
TCR repertoire: TCR antigen-specific diversity produced by V(D)J gene rearrangement during T cell development.
Th1 cytokines: produced by $CD4^+$ helper T cells associated with proinflammatory responses.
Transcriptional regulation: control mechanisms of RNA transcription in response to various signals that coordinate gene expression.
Tumor-associated macrophages (TAMs): myeloid cells associated with the TME that modulate the antitumor response.
Tumor escape: evasion of the immune response by tumor cells owing to loss of immunogenicity or immunosuppressive mechanisms.
Tumor-infiltrating lymphocytes (TILs): immune cells that migrate into the tumor and can potentially recognize and eliminate cancer cells.
Tumor intrinsic resistance: nonresponsiveness to therapy owing to failure of the immune system to elicit an effective antitumor immune response.
Tumor microenvironment (TME): the surroundings of a tumor composed of resident and recruited immune cells, fibroblasts, secreted soluble molecules, blood vessels, and extracellular matrix.

and specific requirements need to be addressed. Candidate epigenetic biomarkers must be tested in homogeneous and reliable patient cohorts (clinical trials). In addition, sensitive and precise detection methods need to be developed to facilitate the discovery of new **epigenetic fingerprints** (see Outstanding Questions). Finally, clinical laboratories must be adapted for marker analysis, which should accompany other prognostic and diagnostic approaches (Box 2).

Epigenetic Modifications That Modulate Antitumor Immunity: Can They Help To Predict Immunotherapy Success?

The sum of accumulated genetic mutations and aberrant changes in the chromatin landscape disrupts cellular homeostasis, and this can lead to cancer initiation and promote tumor progression. Genetic and epigenetic mechanisms cooperate intricately and can act as cancer drivers. Frequent mutations in cancer include those leading to aberrant expression and/or activity of chromatin-modifying enzymes [26].

Tumor mutational burden: the total number of nonsynonymous somatic mutations accumulated by the tumor cell genome.

Whole-genome bisulfate sequencing (WGBS): a next-generation sequencing technique; consists of deep sequencing an entire genome after sodium bisulfite modification to analyze DNA methylation.

Box 2. Laboratory Methods for Analyzing Epigenetic Modifications

Methods for studying epigenetic changes can interrogate a single locus or cover the whole genome.

DNA Methylation

Methylation can be analyzed by methods that include:

(i) Bisulfite conversion of DNA. Genomic DNA is treated with sodium bisulfite, which changes unmethylated cytosines to uracil, whereas 5mC is unchanged. The identification and quantification of modified compared with unmodified cytosines at single loci can be assessed by pyrosequencing. By contrast, DNA methylation arrays and whole-genome bisulfite sequencing (WGBS) – based on high-throughput next-generation sequencing (NGS) – allow genome-wide (with single-base resolution) or whole-genome interrogation, respectively.
(ii) Enzymatic digestion of DNA. This is based on the recognition and fragmentation of genomic DNA by methylation-sensitive restriction enzymes such as MspI, and subsequent analysis of the fragments by NGS.
(iii) Reduced representation bisulfite sequencing (RRBS). Combines enzymatic digestion and bisulfite conversion to generate fragments enriched in CpG-rich regulatory regions, such as promoters, that are then analyzed by parallel sequencing, reducing costs compared with sequencing of the entire genome.
(iv) Affinity-based methods (such as methylated DNA immunoprecipitation sequencing, MeDIP). These are based on enrichment of selected DNA fragments by pulling down genomic DNA with either antibodies or DNA-binding proteins. The enriched fraction is then analyzed by sequencing. One advantage of affinity-based methods is that they allow interrogation of relevant DNA methylation variants such as 5hmC, as in the 5hMeDIP protocol.

Post-translational Histone Modifications

These are mainly assessed by immunoprecipitation with specific antibodies (e.g., against histone modifications or histone variants) to pull down protein–DNA complexes. The DNA is then analyzed by PCR, arrays, or massively parallel sequencing (ChIP-PCR, ChIP-on-ChIP, or ChIP-seq, respectively).

Chromatin Structure and Accessibility

Analysis of these key epigenetic features is informative regarding the molecular mechanisms of pathogenicity. Methods to study accessibility and nucleosome positioning are based on enzymatic digestion and isolation of either nucleosome-bound or accessible regions of DNA coupled to NGS.

Micrococcal Nuclease (MNase)-Seq

Allows interrogation of nucleosome-protected DNA by digesting nucleosome-free genomic regions with MNase from *Staphylococcus aureus*. Undigested DNA represents DNA bound to nucleosomes, which is recovered and sequenced.

Assay for Transposase-Accessible Chromatin (ATAC)-Seq

This method assesses the accessibility of chromatin; it is based on the cutting and addition of adapters to the genomic DNA by Tn5 transposase. In this process, called tagmentation, DNA is amplified by PCR and analyzed by NGS. An open chromatin conformation correlates with the frequency of sequences that map to a given genomic region. Recently, heterogeneity in samples can also be assessed by single-cell ATAC-Seq (scATAC-Seq) protocols that rely on a first step of single-cell separation before ATAC library preparation and sequencing.

Epigenetic Changes in Tumor Cells and Tumor-Associated Immune Cells

The genetic component of tumorigenesis includes mutations that cause activation of oncogenic genes or inactivation of tumor-suppressor genes. However, at the epigenetic level, transcriptional regulation of tumor-associated genes can drive oncogenesis. Cancerous cells often feature increased transcription of **proto-oncogenes** as a result of promoter hyperacetylation [28,29], concomitant with repression of tumor-suppressor genes by promoter hypoacetylation and DNA hypermethylation [30]. Many cancer types show a global loss of DNA methylation throughout the genome, with gains of DNA methylation specifically located at **CpG islands**, thus repressing genes that control cancer growth, such as tumor-suppressor genes [31,32]. Recently, changes in oxidized intermediate 5hmC (5-hydroxymethylcytosine) profiles have been associated with tumor aggressiveness and a less-differentiated phenotype, both in KRAS mutant mouse models of pancreatic ductal adenocarcinoma (including *Pdx1-cre*;*LSL-Kras*^{G12D};*Col1a1-TRE-shp53-shRenilla*;*Rosa26-CAGs-LSL-rtTA-IRES-mKate2*) [33] and in some human hematological malignancies such as acute myeloid leukemia (AML) [34].

Accompanying extensive modification of the tumor cell epigenome, reconfiguration of the TME and tumor-driven rewiring of immune cell chromatin landscapes can also modulate the extent and quality of the antitumor immune response, the potential response to immunotherapy, and overall disease outcome. A protumorigenic immunosuppressive TME epigenetically affects immune cells, and can lead to **tumor escape** [28,35]. From the extent of cellular accumulation of TILs in the TME, tumors may be arbitrarily classified as 'hot', when highly or modestly infiltrated, or 'cold', if they are noninfiltrated tumors [36]. Inflamed 'hot' TMEs are compatible with effective type 1 T helper (Th1) cell IFN-γ-mediated antitumor immune responses, as shown by the correlation between the presence of an IFN-responsive genes signature in some tumors (e.g., melanoma, and head and neck squamous cell carcinoma, HNSCC), and a better response to immunotherapy, compared with tumors lacking the IFN-γ signature [37]. For the above reasons, it is crucial to understand how tumors shape an immunosuppressive TME. TILs, particularly cytotoxic CD8$^+$ T cells (CTLs), often display an 'exhausted' phenotype resulting from persistent antigen stimulation [38,39]; an immunosuppressive TME can contribute to tumor-specific CTL exhaustion, as shown in a mouse model of tamoxifen-inducible liver cancer (*ASTxCre-ER*^{T2}) [38]. CD8$^+$ T cell exhaustion features specific chromatin-accessible regions associated with an altered transcriptional profile, including enrichment for genes such as *Pdcd1* (encoding PD-1), and the *Ifng* and *Il10* signaling pathways [40]. Finally, concomitant extensive epigenetic reprogramming [41] leading to silencing of genes involved in effector differentiation, such as *Ifng*, *Tcf7*, *Myc*, and *Ccr7*, have resulted in loss of CD8$^+$ T cell effector function in mice [42,43] (Figure 1). Of relevance, anti-PD-1 Ab therapy can partially reverse CD8$^+$ T cell exhaustion, but achieving a durable antitumor CD8$^+$ T cell effector response has proved challenging [44]. Chromatin states can differentiate dysfunctional tumor-infiltrated PD-1-positive CD8$^+$ T cells, whose exhaustion might be reversed, from those that are potentially refractory to anti-PD-1 Ab therapeutic intervention [45]. Tumor antigen-specific CD8$^+$ T cells are rendered dysfunctional during tumorigenesis in a tamoxifen-inducible hepatocellular carcinoma murine model (*ASTxCre-ER*^{T2}), and they fail to produce IFN-γ and tumor necrosis factor (TNF)-α as a result of chromatin remodeling, as evidenced from ATAC (assay for transposase-accessible chromatin)-seq data [45]. Such epigenetic reconfiguration can be reverted by *ex vivo* treatment with IL-15 [45]. Similar chromatin-accessibility profiles have been described in human TILs from melanoma and non-small cell lung tumors (NSCLC) [45]. From another angle, double-stranded RNAs (dsRNAs) can accumulate in cancer cells upon derepression of endogenous retroviruses (ERVs) by **epigenetic drugs** such as DNA demethylating agents and lysine demethylase 1 (LSD1) inhibitors in ovarian cancer cell lines [46]. This is relevant because cytoplasmic accumulation of dsRNAs mimics viral infection, triggering an IFN response within the TME and promoting robust antitumoral immunity [46]. In cell lines derived from hematological cancers and ovarian cancer, dual

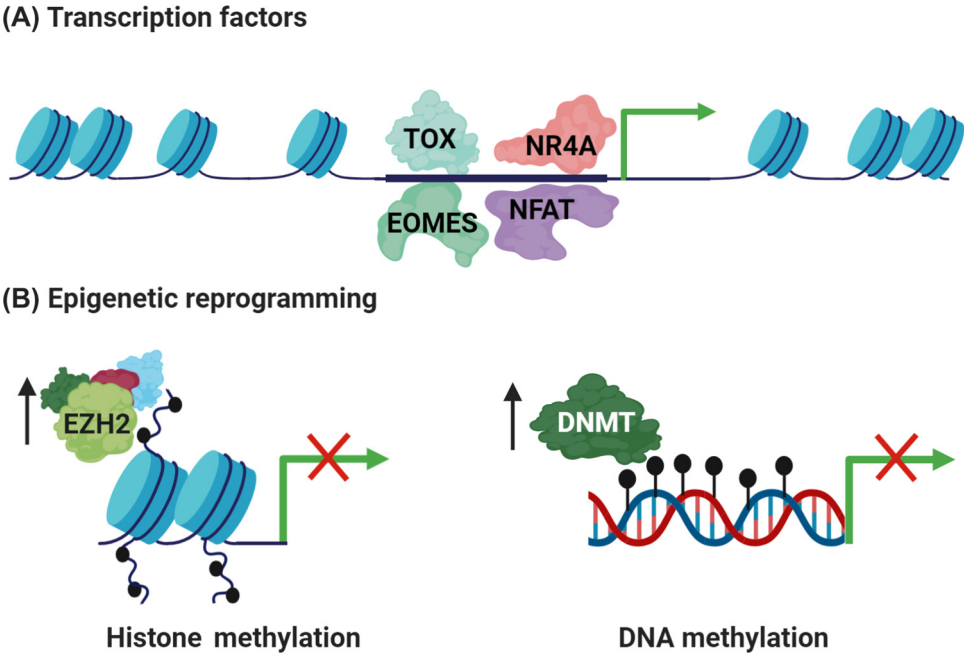

Figure 1. Intrinsic Mechanisms Driving CD8$^+$ T Cell Exhaustion in Cancer. In addition to immunosuppressive factors in the tumor microenvironment (TME), transcriptional regulation and epigenetic restraining through DNA methylation and repressive histone marks can induce a dysfunctional CD8$^+$ T cell state. (A) An increase in transcription factors such as TOX, NRA4, EOMES, and NFAT, that upregulate the expression of inhibitory receptor genes, including *Pdcd1*, *Havcr2*, and *Lag3* (which encode PD-1, TIM3, and LAG3) among others, is associated with a terminally 'exhausted' phenotype [40,42,100]. (B) EZH2, part of the PRC2 H3K27me3 chromatin writer complex, is a key regulator of transcriptional silencing of memory-associated genes that leads to terminally differentiated effector CD8$^+$ T cells [41]. This repressive mark has been associated with tumor-specific CTL dysfunction in pre- and early malignant lesions [38]. DNA methylation enzymes, such as DNMT1 and DNMT3B, are upregulated in exhausted CD8$^+$ T cells [38] and DNMT3A genome-wide *de novo* methylation can promote terminal exhaustion [43]. Black arrows represent an upregulation of epigenetic enzymes recruitment and red crosses represent transcriptional repression. This figure was created using BioRender (https://biorender.com/). Abbreviations: CTL, cytotoxic T lymphocyte; DNMT, DNA methyltransferase; H3K27me3, histone H3 trimethylated on lysine 27.

inhibition of methyltransferase G9a and DNA methyltransferases (DNMTs) caused upregulation of nonoverlapping groups of ERV transcripts, with subsequent induction of viral defense genes such as those encoding IRF7 and STAT1 [46,47]. In colorectal cancer initiating cell lines (CICs), treatment with the demethylating agent 5-AZA (5-aza-2-deoxycytidine) induces IFN response factors such as IRF7 and OASL by prompting dsRNA upregulation and activating the MDA5/MAVS/IRF7 signaling pathway [48]. Thus, targeting of cancer cells with epigenetic drugs can induce viral mimicry leading to activation of IFN responses that promote effective immune responses; these, if combined with checkpoint inhibition therapy, might potentially enhance antitumoral responses and increase therapeutic success.

Epigenetic Modifications as Putative Biomarkers for Immunotherapy

Several preclinical studies in mice, cell lines, and patient samples propose potential epigenetic biomarkers whose fingerprints are associated with immune evasion or response signatures that can be used as indicators of immunotherapy efficacy (Table 1 and Outstanding Questions). Of note, most studies to date describe biomarkers in bulk tissue preparations, and further information regarding biomarkers at the cellular level is still missing. **Deconvolution analysis** of transcriptomic and epigenomic data from tumor tissues is a potent tool for identifying the cell types that express dysregulated genes [49]. Indeed, DNA methylation-based deconvolution

Table 1. Potential Epigenetic Biomarkers Corresponding to an Immunocompetent Phenotype

Putative biomarker	Mark	Source	Malignancy	Refs
$CD8^+$ T cell infiltration	H3K27me3 at Th-1 genes	Mouse	Ovarian cancer	[35]
	Lysine demethylase 1 (LSD1) inhibition	Mouse	Melanoma	[51]
	H3K27me3 at *CXCL9*	Human Mouse	Glioblastoma Ovarian cancer	[52]
	CCL5 methylation	Human, mouse	Melanoma, breast, ovarian, colon, and lung	[53]
	Nuclear receptor-binding SET domain protein 1 (NSD1) inactivation	TCGA Human cell lines Mouse	HNSCC	[54]
	PDCD1LG2 methylation	TCGA Singapore cohort	Gastric adenocarcinoma	[55]
$CD8^+$ T cell exhaustion	*PRF1* methylation	Human (*ex vivo*)	Urothelial bladder cancer (UBC)	[63]
	$CD8^+$ T cell genome-wide methylation	Mouse	Pan-cancer	[43]
Immunological synapse and antigen presentation	H3K27me3 H3K27ac	Human	Gastric adenocarcinoma	[64]
	H3K27me3	Mouse	Melanoma	[57]
	H3K27me3 and H3K27ac at *MHCI*	Human Human cell lines Mouse	Small cell lung cancer (SCLC)	[58]
	HLA, *CD40*, *CD80*, *CD86* methylation	TCGA	Pan-cancer	[56]
Novel ICB treatments	*ADORA2A* methylation	TCGA	HNSCC	[61]
	TNFRSF9 methylation	Human	Melanoma	[62]

analysis revealed that aberrant activation of the transforming growth factor (TGF)-β pathway in cancer-associated fibroblasts could drive immunosuppression in tumors that were originally 'hot', thus preventing a successful response to ICB [50]. Epigenetic silencing by EZH2-mediated histone methylation and DNMT1 DNA methylation can constitute tumorigenic mechanisms. In whole-tissue studies, inhibition of the epigenetic modifiers EZH2 and DNMT1 in tumor cells from an ovarian cancer ID8 model in mice increased the expression of Th1 chemokine genes *Cxcl9* and *Cxcl10* by the tumor [35]. Combined epigenetic treatment and immunotherapy promoted increased $CD8^+$ T cell infiltration and improved the anti-PD-L1 Ab therapeutic effect compared with immunotherapy in the absence of EZH2 and DNMT1 inhibition [35]. Thus, epigenetic modulation may revert the immune evasion caused by epigenetic silencing of Th1 chemokines [35]. Moreover, histone demethylase LSD1 genetic ablation in mouse syngeneic melanoma tumor cells promoted $CD4^+$ and $CD8^+$ T cell tumor infiltration and increased tumor cell immunogenicity in mice bearing LSD1-deficient B16 tumors, relative to their wild-type (WT) counterparts [51]. Furthermore, leukemia inhibitory factor (LIF)-mediated epigenetic silencing of *Cxcl9* in TAMs isolated from an ID8 ovarian cancer mouse model correlated with reduced $CD8^+$ T cell tumor infiltration and limited anti-PD-1 Ab efficacy upon LIF blockade [52]. In human ovarian cancer, *CCL5* and *CXCL9* overexpression leads to $CD8^+$ T cell tumor infiltration and longer patient survival and response to anti-PD-1 Ab ICB compared with nonoverexpressing cells [53]. In addition, epigenetic silencing of *Ccl5* by DNA methylation in ID8 mouse ovarian cancer tissue led to impaired TIL recruitment and CXCL9 secretion by TAMs, as evidenced from immunostaining and multispectral microscopy in Cxcl9-expressing tumors compared with $Cxcl9^{low}$ tumors, yielding 'cold' tumors that decrease animal survival and the response to anti-

PD-1 Ab [53]. In addition, The Cancer Genome Atlas (TCGA) identified a subtype of HNSCC, characterized by inactivation of histone methyltransferase nuclear receptor-binding SET domain protein 1 (NSD1), as well as by DNA hypomethylation, showing a 'cold' tumor phenotype with low immune cell infiltration as well as low PD-1 expression in $CD8^+$ T cells, relative to active NSD1-expressing tumors [54]. The authors also demonstrated in a HNSCC xenograft mouse model that *Nsd1* knockdown in tumor cells resulted in downregulation of chemoattractant cytokines and tumor immune desertification relative to control transduced WT tumors [54]. Hence, epigenetic mechanisms that promote tumor recruitment of lymphocytes might synergize with subsequent ICB immunotherapy. The epigenetic status of *PDCD1LG2* (encoding PD-L2) in cancer cells also appears to be relevant, at least in some tumor types; specifically, methylation of several CpGs within this locus have been associated not only with $CD8^+$ T cell tumor infiltration but also with a high tumor mutational burden in patients with gastric adenocarcinomas [55]. From another perspective, analysis of TGCA DNA methylation data from 20 human solid tumors revealed that hypomethylation of **immunological synapse** genes such as *HLA*, *CD40*, and *CD80* in immunogenic tumors such as melanoma correlated with effector $CD4^+$ and $CD8^+$ T cell infiltrates modulating the tolerogenicity of the TME [56]. Three different murine models of melanoma (B16-F10, RIM3, and $Nras^{Q61K}Ink4a^{-/-}$) have demonstrated upregulation of Ezh2 expression on tumors upon anti-CTLA-4 Ab treatment [57]. Such upregulation results in the epigenetic repression of tumor immunogenic genes, such as MHC-I genes and *Cxcl9*, reducing antigen processing and presentation to immune cells relative to controls [57]. Accordingly, whole-genome CRISPR/Cas9 screening in the erythroleukemia cell line K562 showed a repressive role of PRC2 on MHC-I genes in tumor cells, leading to tumor growth and resistance to CTL-mediated tumor cell killing in a murine model of small-cell lung cancer (SCLC). Moreover, EZH2 inhibition allowed restoration of HLA gene expression [58]. In another study, ATAC-seq on sorted IFN-γ-treated melanoma cells revealed an increase of open chromatin regions relative to untreated cells, leading to higher STAT-1 occupancy on loci such as *MHC-II* and *CD274*, and driving transcriptomic features of resistance to anti-CTLA-4 Ab treatment [59]. Indeed, the search for novel, robust, and valid ICB targets is an active field of research. Molecules with immunomodulatory effects, such as Adora 2A and galectin 3, can be epigenetically controlled in some cancers including prostate and HNSCC, respectively [60], and are being proposed as potential biomarkers for patient stratification. Specifically, low promoter *ADORA2A* DNA methylation in tumors from HNSCC patients correlated with immune cell infiltration and overall survival (OS) [61]. Within the immune compartment, $CD8^+$ T cells show epigenetic signatures that might help to determine their antitumoral potential upon ICB therapy. For instance, the T cell co-stimulatory receptor TNFRSF9 (also known as 4-1BB/CD137), whose expression is regulated by DNA methylation, may be a promising therapeutic target for melanoma treatment [62]. A recent study in a TCGA cohort of cutaneous melanoma showed that DNA methylation status of the human *TNFRSF9* promoter correlated with the extent of lymphocyte (including NK cell) and macrophage infiltration into the tumor [62]. Owing to the regulatory role of DNA methylation on *TNFRSF9* expression in tumor-infiltrating immune cells, and given that TNFRSF9 promotes immune activation, these findings suggest a layer of epigenetic regulation of TNFRSF9 expression that contributes to enhanced lymphocyte recruitment and activation by tumors, thereby promoting antitumoral responses [62]. Finally, increased expression and promoter hypomethylation of *TNFRSF9* correlated significantly with higher progression-free survival (PFS) and robust response to anti-PD-1 Ab therapy in melanoma patients, supporting its possible suitability as a predictive biomarker of the response to immunotherapy [62].

Analysis by **whole-genome bisulfate sequencing (WGBS)** of isolated virus-specific $CD8^+$ T cells from a mouse model of chronic infection with lymphocytic choriomeningitis virus (LMCV) strain clone 13 revealed the acquisition of exhaustion-associated DNA-methylation programs through *de novo*

DNMT3a-mediated methylation of pivotal genes such as *Ifng*, *Myc*, *Tcf7*, *Ccr7*, and *Tbx21* [43]. The same differentially methylated regions were observed in tumor-infiltrating PD-1^{high}Tim3^{+}CD8^{+} T cells isolated from a TRAMP-C2 prostate tumor mouse model. Of relevance, combined PD-L1 blockade and treatment with DNA demethylating agents reverted the exhaustion program and induced proliferation of tumor-infiltrating PD1^{+}CD8^{+} T cells, thus reducing tumor growth in these mice compared with PD-L1 monotherapy [43]. Recently developed single-cell technologies allowing the interrogation of the heterogeneity of complex samples are shedding light on the contributions of different cell types to cancer responses to immunotherapy. In one study, single-cell transcriptomic analysis of CD8^{+} TILs revealed two cellular subsets, progenitor and terminally exhausted, that differed in their chromatin accessibility and transcription of *Slamf6* and *Havcr2* (encoding Tim-3), and which largely determined the cytotoxic capacity of these cells in response to PD-1 blockade in mice bearing B16-OVA tumors [40]. In another study, DNA methylation of sorted exhausted CD8^{+} tumor tissue-resident memory T cells (Trms) from urinary bladder cancer patients was interrogated. Tumor-infiltrating Trm cells showed that low DNA methylation in an enhancer region of the *PRF1* locus correlated with high expression of the perforin protein, and allowed restoration of Trm antitumoral cytotoxic activity upon TCR *ex vivo* stimulation in the presence of IL-15 [63]. Furthermore, single-cell transcriptional profiling identified a population of dysfunctional PD-1^{high}CD8^{+} T cells with reduced chromatin accessibility in *TCF7* regions; these cells were associated with exhaustion and a failed response to checkpoint blockade in nonresponder melanoma patients relative to responders [42]. Such evidence may support the rationale for using epigenetic 'biomarkers' and transcriptional programs to delineate therapeutic strategies that might drive or complement anticancer treatments [42].

Epigenetic modifications relevant to the immunotherapy response have also been identified in patients treated with immunotherapeutic agents (Table 2). Comparison of histone modification profiles [histone H3 trimethylated or monomethylated on lysine 4 (H3K4me3, H3K4me1) or acetylated on lysine 27 (H3K27ac)] in promoters of human primary gastric tumors, gastric cancer cell lines, and normal gastric tissue revealed altered chromatin profiles in somatic promoters that led to dysfunctional antigen presentation and tumor depletion of immunogenic peptides, hence reducing tumor antigenicity, strongly suggesting an epigenetically controlled mechanism of immune evasion of immunotherapy [64]. Early studies showed that tumor *PDCD1LG1* (coding for PD-L1) promoter methylation was associated with anti-PD-1 Ab resistance in patients with NSCLC refractory to treatment with EGFR tyrosine kinase inhibitors, and who were treated with nivolumab (anti-PD-1 Ab) [65]. The value of unambiguously assessing epigenetic biomarkers to facilitate the prediction of response to immunotherapy was first demonstrated in matched pretreatment and post-ICB samples in a cohort of melanoma patients. Specifically, lower *CTLA4* methylation in malignant melanoma tissue samples from a TGCA cohort was associated with a stronger response to anti-PD-1 and

Table 2. Epigenetic Hallmarks Identified in a Cohort of Cancer Patients Treated with Immunotherapy

Epigenetic mark	Malignancy	Treatment	Outcome	Refs
PD-L1 methylation	NSCLC	Anti-PD-1 Ab	Anti-PD-1 Ab resistance	[65]
Low CTLA-4 methylation	Melanoma	Anti-PD-1 Ab Anti-CTLA-4 Ab	Response to therapy and overall survival	[66]
H3K27ac/H3K4me3	Gastric cancer	Anti-PD-1 Ab	Resistance to anti-PD-1 Ab	[70]
TET2 disruption	CLL	CART19 cell	Enhanced CAR-T cell activity and complete remission	[68]
FOXP1 demethylation and EPIMMUNE epigenetic signature	NSCLC	Anti-PD-1 Ab	Clinical benefit with PD-1 blockade	[27]
Global low methylation	Lung cancer	Anti-PD-1 Ab	Poor prognosis and lower tumor immunity	[67]

anti-CTLA-4 Ab therapy and increased OS compared with the non-ICB cohort [66]. Subsequent studies have highlighted the role of epigenetics in predicting or modulating the immune response to immunotherapy. A multicenter study conducted in tumor tissue from NSCLC patients treated with anti-PD-1 Ab compared the presence and absence of an epigenetic signature (named EPIMMUNE), and showed that the presence of EPIMMUNE was associated with improved progression-free survival (PFS) and OS, thus proposing EPIMMUNE as a good predictor of immunotherapy response [27]. A second independent study described a global DNA-methylation signature in TGCA tissue samples of lung cancer and melanoma that correlated loss of DNA methylation with tumoral $CD8^+$ T cell infiltration, revealing poor prognosis associated with weaker tumor immunity that was independent of mutation burden [67]. Moreover, disruption of the ten-eleven translocation methylcytosine dioxygenase 2 (*TET2*) gene in a single clone of CAR-T cells was associated with enhanced cytotoxic activity of a CAR-T against CD19 antigen (CART19) upon *ex vivo* restimulation. Infusion of CART19 in a CLL patient showed clonal expansion of CART19 cells and a potent antitumoral response that led to complete remission in that patient [68] (see Outstanding Questions). These observations emerged from a completed nonrandomized pilot/Phase I study (single-arm) in patients with $CD19^+$ leukemia and lymphoma (NCT01029366[ii]). Patient outcome correlated with vector integration site, including proximity to epigenetic chromosomal features such as the repressive mark H4R3me2 and the chromatin hyperacetylation-associated gene *BRD3* [69]. In another example, transcriptomic data revealed that the extent of alternative promoter usage at 2732 sites in gastric cancer biopsies correlated with immune evasion and resistance to anti-PD-1 Ab immune checkpoint inhibition in gastric cancer patients [70], suggesting that alternative promoter usage might have potential as a predictive biomarker for immunotherapy.

In conclusion, the leverage of epigenetic changes controlling tumor-associated immune responses to reinforce immunotherapy may be a promising route for therapeutic intervention in cancer patients and certainly deserves further robust investigation because many unknowns remain, particularly when attempting to predict responses across different tumor types. In particular, given the need to associate reliable biomarkers with immunotherapy success, epigenetic changes that might accompany and/or be predictive of treatment outcomes are desirable, and may be relevant to the development of precise diagnostic and stratification strategies (Figure 2).

Epigenetic Drugs To Boost Immunotherapy-Based Strategies

The highly dynamic nature of epigenetic regulation has been harnessed from a therapeutic point of view through the development of drugs (epidrugs) that target the chromatin-modifying machinery and affect epigenetic changes (Box 3). The reversibility of epigenetic modifications has enabled pharmacological interventions with DNA hypomethylating agents (HMAs) and histone deacetylase inhibitors (HDACis).

Clinical and Preclinical Trials Combining Epidrugs and Immunotherapy

A challenging aspect of cancer management is the development of resistance to treatment, and this is a major limitation of many anticancer therapeutic approaches. The immunomodulatory effect of epigenetic drugs can be leveraged to enhance the antitumor effect of immunotherapy, as shown in transplantable murine models of mammary carcinoma and mesothelioma where combining the DNA HMA 5-aza-2′-deoxycytidine (5-AZA-CdR) with an anti-CTLA-4 monoclonal Ab led to increased antitumor activity relative to controls [71]. This study set the rationale for a single-arm Phase Ib trial using the DNA HMA guadecitabine combined with ipilimumab (anti-CTL4 Ab) in patients with unresectable stage III/IV melanoma (NCT02608437[iii]). Treatment safety and tolerance were assessed in the trial, together with treatment-related immunomodulatory effects including activation of immune-related pathways, upregulation of HLA-I by melanoma cells, and increased numbers of $CD8^+PD\text{-}1^+$ T cells and $CD20^+$ B cells at the tumor site post-treatment

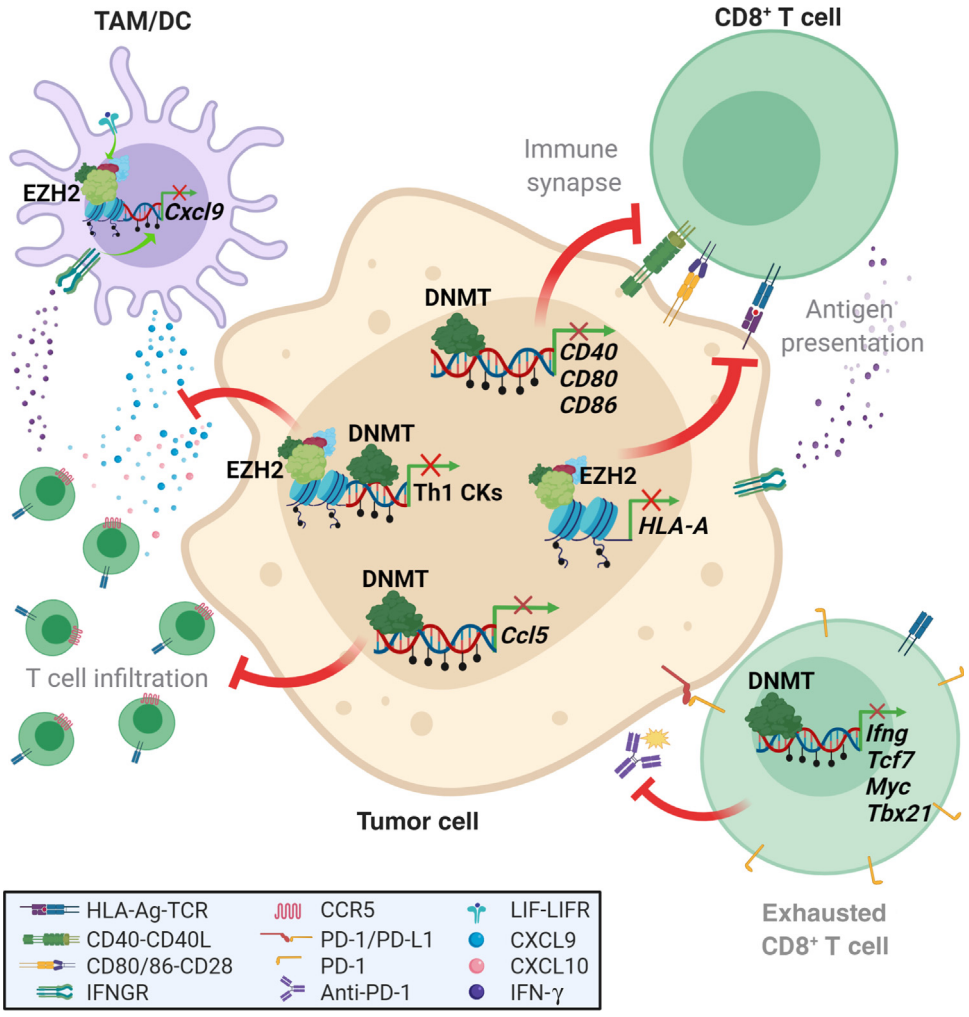

Figure 2. Epigenetic Mechanisms in the Tumor Microenvironment (TME) That Are Relevant to Cancer Immunotherapy. DNA and histone methylation can shape the TME by regulating immune-related gene expression. DNA and histone methylation by DNMT1 and EZH2, respectively, result in downregulation of the chemoattractant cytokine genes *Cxcl9* and *Cxcl10*, thereby dampening $CD8^+$ T cell recruitment to mouse ovarian tumors [35]. Interferon (IFN)-γ-induced Cxcl9 secretion by antigen-presenting cells (APCs) is impaired because of decreased $CD8^+$ T cell infiltration upon *Ccl5* methylation in tumor cells [53]. Leukemia inhibitory factor (LIF)-mediated EZH2 recruitment to the *Cxcl9* promoter triggers its epigenetic silencing in tumor-associated macrophages (TAMs) [52]. Immunological synapse formation and antigen presentation, which are necessary for an effective antitumor cytotoxic response, may be epigenetically silenced in tumor cells by methylation [58]. Finally, methylation of genes in programmed cell death 1 $(PD\text{-}1)^+CD8^+$ T cells can induce an 'exhausted' state that is refractory to effector response rescue with anti-PD-1 antibody therapies [43]. Red crosses represent transcriptional repression. This figure was created using BioRender (https://biorender.com/). Abbreviations: CK, cytokine; DC, dendritic cell; DNMT, DNA methyltransferase; Th1, type 1 T helper cell.

relative to baseline [72]. Treatment with hypomethylating drugs has also been shown to cause upregulation of *CD274*, *PDCD1LG2*, and *CTLA4* in myelodysplastic syndrome (MDS) and AML $CD34^+$ cells; patients resistant to treatment with the demethylating agent decitabine showed higher expression of such genes relative to sensitive patients [73]. In addition, *PDCD1* promoter demethylation in $CD4^+$ and $CD8^+$ T cells from MDS or AML patients after 5-AZA treatment tended to show a poorer response to treatment and shorter overall response (OR) than patients with a methylated *PDCD1* promoter [74]. This has established a rationale for

Box 3. Epidrugs Currently Accepted for Clinical Use

DNMT Inhibitors

Hypomethylating agents (HMAs) currently approved for clinical use are the nucleotide analogs 5-aza-2-deoxycytidine (5-AZA) and decitabine (5-AZA-2′-deoxycytidine, 5-AZA-CdR). These molecules are incorporated into the DNA of dividing cells, causing irreversible binding and degradation of DNMTs, and subsequent activation of DNA damage response (DDR) pathways [91]. The activation of the DDR triggers **apoptosis** of highly proliferative cells, promoting cytotoxicity in addition to the epigenetic effect of HMAs.

HDAC Inhibitors

Vorinostat (suberoylanilidehydroxamic acid, SAHA) inhibits the enzymatic activity of class I and II HDACs [92]. It was first approved by the FDA in 2006 for the treatment of progressive, persistent, or recurrent (relapsed/refractory) cutaneous T cell lymphoma (CTCL) [93]. Romidepsin (cyclic peptide and depsipeptide) blocks HDAC activity, causing apoptosis. It was approved by the FDA for CTCL in 2007 and for peripheral T cell lymphoma (PTCL) in 2011. The hydroxamate-type class I, II, and IV HDAC inhibitor belinostat was approved for relapsed or refractory PTCL in 2014. Finally, in 2015, the FDA approved panobinostat, a nonselective pan-HDAC inhibitor (in combination with bortezomib and dexamethasone) for the treatment of multiple myeloma (MM).

Emerging Epigenetic-Based Therapies

Drug discovery devoted to the search for novel compounds that specifically target or reverse cancer-promoting epigenetic modifications is a fast-expanding field. Upon screening with a library of several epigenetic targeting compounds, an aurora kinase inhibitor has been shown to kill thyroid cancer cell lines efficiently, making it a potentially strong candidate for novel epigenetic treatment of this cancer type [94]. A newly developed mutant IDH1 inhibitor (DS-1001b) has shown promising antitumor properties in chondrosarcoma cell lines [95]. Ricolinostat, the first selective HDAC6 inhibitor [96], is currently being tested in a Phase Ib/Phase II clinical trial to treat MM in combination with pomalidomide and dexamethasone. This ongoing open-label trial recruited a total of 101 patients, with one study arm to assess safety and best dose (Phase Ib) as well as overall response to treatment (Phase II) in relapsed and refractory MM (NCT01997840[xiii]) Other small molecules that selectively inhibit HDAC6 are showing greater efficacy and promising results in preclinical settings [97,98]. Novel experimental approaches to promote targeted epigenome editing, such as directed integration of CpG-free DNA to induce *de novo* DNA methylation and to allow the correction of abnormal epigenetic modifications on disease-causing loci, may have therapeutic potential and warrant further investigation [99].

Outstanding Questions

Are we putting the 'CART' before the horse? Is it necessary to further characterize the human epigenome before immunotherapy administration because treatment itself may cause epigenetic alterations in tumor cells, and because most immunotherapy treatments are administered as second-line therapy? Could this new knowledge contribute to new combinatorial strategies to overcome immunotherapy resistance?

Is it worth studying DNA methylation changes in patient-derived $CD8^+$ T lymphocytes induced by immunotherapy as a means of monitoring biomarkers of immunotherapy response?

Should efforts be devoted to identifying unique universal biomarkers for each type of cancer, or should they instead be devoted to defining 'response signatures' for patient stratification?

How much do epigenetic tests reflect tumor heterogeneity? Does it make more sense to aim for single-cell resolution? The TME is important in treatment resistance, but cell clonality could be the key to understanding specific epigenetic changes that drive treatment failure or success. Could this provide a rationale for target insertion strategies in the design of CAR-T cell therapies?

Will technology allow the use of epigenetic biomarkers in routine clinical settings to predict or monitor immunotherapy response? It is still necessary to simplify the methods and decrease the costs of measuring epigenetic biomarkers.

Is the medical community prepared to adopt the use of epigenetic biomarkers as a means to develop precision medicine? How can the use of epigenetic biomarkers be exploited so that these are translated from the bench to the bedside?

evaluating the success of combinatorial therapy with HMAs and ICB in AML and MDS patients [75,76]. Moreover, treatment with CDK4/6 inhibitors reduced DNMT1 mRNA and protein expression, causing hypomethylation of immune-related genes (such as those encoding STATs and IRFs), in mouse tumor models (*MMTV-rtTA/tetO-HER2*, *MMTV-PyMT*) and patients with breast and colon carcinoma, enhancing antitumor immunity by both promoting antigen presentation and reducing Treg cell expansion relative to controls [77]. The combination of CDK4/6 inhibitors with anti-PD-L1 Ab therapy in these mouse models led to tumor regression and resistance to rechallenge with tumoral cells compared with untreated mice [77].

In line with this, a novel dual inhibitor that targets both G9a and DNMT has been used in combination with anti-PD-L1 Ab therapy in a mouse transgenic model of bladder cancer ($Pten^{loxP/loxP}$; $Trp53^{loxP/loxP}$; $Rb1^{loxP/loxP}$; $Rbl1^{-/-}$), and enhanced the antitumor response by increasing $CD8^+$ T cell and NK cell tumor infiltration and inducing tumor regression relative to both untreated mice and anti-PD-L1-Ab-only treated mice [78]. In the case of HDAC inhibitors, entinostat is a synthetic benzamide that selectively inhibits class I and IV HDACs and is being investigated in clinical trials in combination with anti-PD-1 Ab therapy (pembrolizumab) for colorectal cancer and ICB-resistant melanoma [79–81] and NSCLC [82–84]. Figure 3 summarizes selected ongoing clinical trials.

Concluding Remarks

Immunotherapy treatments have led to a major breakthrough on the road to monitoring, controlling, and eliminating particular malignancies. However, because every cancer is unique, efforts must be concentrated on disentangling the way in which each tumor type manifests at the molecular level and, consequently, how treatments can affect the development and potential

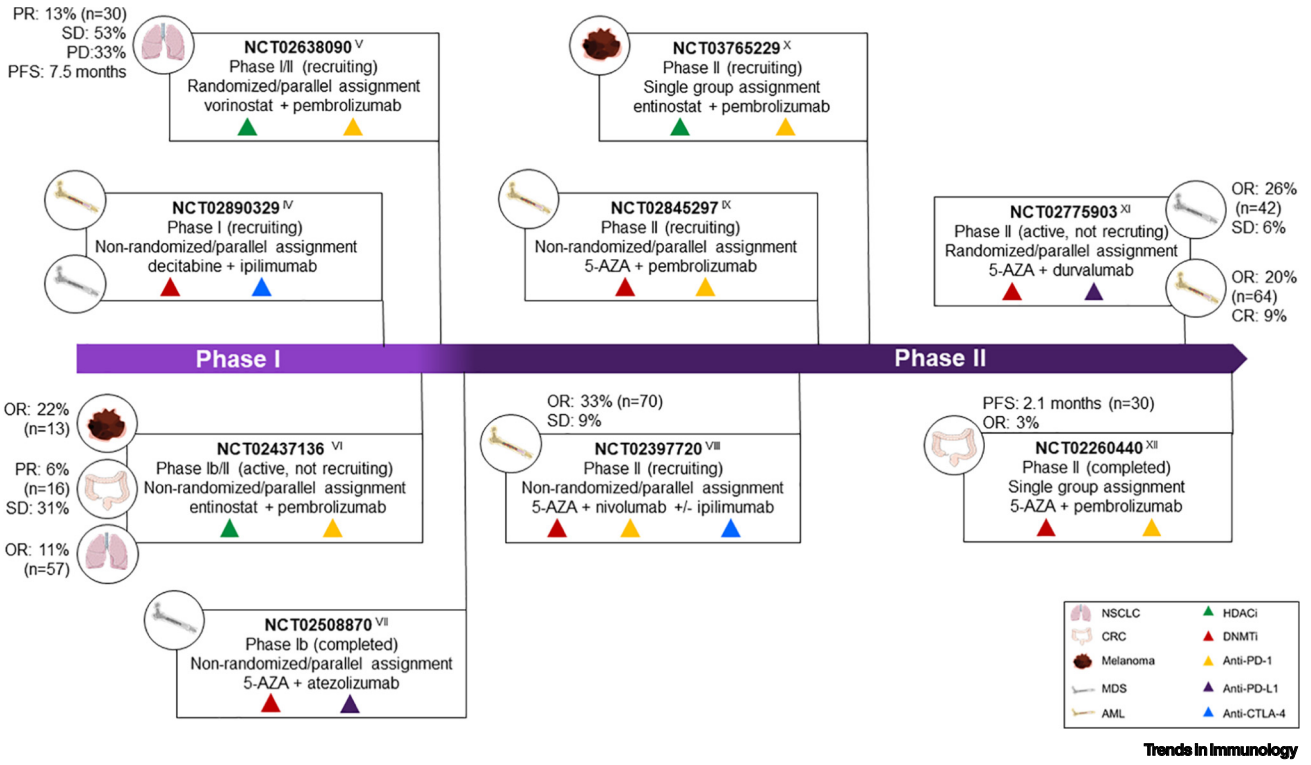

Figure 3. Timeline of the Combinatorial Use of Epigenetic Drugs with Immunotherapy in Clinical Trials. Examples of ongoing clinical trials using different combinations tested for solid tumor and hematological malignancies are shown. Drug names of DNA methyltransferase inhibitors (DNMTis) and histone deacetylase inhibitors (HDACis), as well as immune checkpoint inhibitor molecules, are shown. Cancer types treated in the trial are illustrated with respect to the affected organ. The state of the trial (recruiting, active, or completed) is indicated. Overall response (OR), progression-free survival (PFS), partial response (PR), and stable disease (SD) are shown for each clinical trial according to published data (NCT02890329[iv], NCT02638090[v], NCT02437136[vi], NCT02508870[vii], NCT02397720[viii], NCT02845297[ix], NCT03765229[x], NCT02775903[xi], NCT02260440[xii]) [75,76,79–81,83]. Abbreviations: AML, acute myeloid leukemia; 5-AZA, 5-aza-2-deoxycytidine; CRC, colorectal cancer; CTLA-4, cytotoxic T-lymphocyte antigen 4; MDS, myelodysplastic syndrome; NSCLC, non-small cell lung cancer, PD-1, programmed cell death 1.

eradication of each individual tumor. This knowledge must be translated into updated medical guidelines if specific and effective therapies for different cancer types are to be deployed. One way to implement personalized medicine for cancer relies, in part, on identifying robust biomarkers that might not only predict response to immunotherapy but also factor in individual differences and dynamic changes caused by the disease course itself and/or by the treatment. In this respect, epigenetic biomarkers, including epigenetic signatures, might represent sensitive predictors of the predisposition of each patient for response to treatment. As such, they might offer valuable mechanistic information to potentially prevent or interfere with a desired therapeutic response. Nonetheless, epigenetic and novel immunotherapy treatments are both nascent tools in clinical practice, and further research will be necessary to discover and develop reliable epigenetic biomarkers. By leveraging this knowledge, new-generation epigenetic drugs suitable for combining with immunotherapy might be designed (see Outstanding Questions). We emphasize that the availability of effective epigenetic biomarkers and new combinatorial approaches are likely to increase the chances of success in cancer therapeutics.

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Disclaimer Statement

M.E. is a consultant for Ferrer International and Quimatryx.

Resources

[i] https://clinicaltrials.gov/ct2/show/NCT03056339
[ii] https://clinicaltrials.gov/ct2/show/NCT01029366
[iii] https://clinicaltrials.gov/ct2/show/NCT02608437
[iv] https://clinicaltrials.gov/ct2/show/NCT02890329
[v] https://clinicaltrials.gov/ct2/show/NCT02638090
[vi] https://clinicaltrials.gov/ct2/show/NCT02437136
[vii] https://clinicaltrials.gov/ct2/show/NCT02508870
[viii] https://clinicaltrials.gov/ct2/show/NCT02397720
[ix] https://clinicaltrials.gov/ct2/show/NCT02845297
[x] https://clinicaltrials.gov/ct2/show/NCT03765229
[xi] https://clinicaltrials.gov/ct2/show/NCT02775903
[xii] https://clinicaltrials.gov/ct2/show/NCT02260440
[xiii] https://clinicaltrials.gov/ct2/show/NCT01997840

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